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TITLE: Expression and Significance of CYR61 Expression in Breast  
Cancer Tumor Specimens

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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> Breast cancer often progresses from an estrogen (E2)-dependent, non-metastatic, antiestrogen-sensitive phenotype to an E2-independent, antiestrogen-resistant, highly invasive, and metastatic phenotype. To develop this aggressive phenotype, cells must circumvent normal tissue constraints and lose normal regulation of genes that control tissue homeostasis. Several prognostic markers in human breast cancers have been reported. Prediction of breast cancer recurrence can be assessed based on the size of the tumor, number of lymph nodes involved, estrogen receptor (ER) and progesterone receptor (PgR) status, and histological grade. It is known that adjuvant therapy improves long-term survival in breast cancer patients. In contrast to the invasive ER-negative breast carcinomas, the axillary node-negative (ANN) breast cancer patients have a good prognosis; nevertheless, there is still an appreciable relapse rate. In general, adjuvant treatments benefit some patients with node-negative disease. However, large numbers of women (including those in whom no recurrence will occur) are treated in order to benefit those who will relapse. Thus, to reduce the toxicity and improve the efficacy of the drugs, we must identify appropriate adjuvant treatments for different groups of patients, and we should be able thereby to improve our predictions regarding which group of patients will benefit a treatment. We have identified Cyr61, a member of a family of cysteine-rich secreted proteins. Cyr61 binds to the $\alpha v \beta 3$ integrin and mediates cell attachment, adhesion, migration, extracellular matrix signaling, and angiogenesis. Cyr61 is expressed in all of the tumorigenic and metastatic breast cancer cell lines. We have shown that Cyr61 promotes antiestrogen resistance and that Cyr61 is expressed in 30% of human breast carcinomas, and that its expression correlates with HRG-expression and with the lack of ER expression. Taking into consideration that administration of Tam to breast cancer patients with ANN induces a substantial regression of the tumor and an increase in disease-free survival, together with our knowledge that Cyr61 expression correlates with antiestrogen resistance, it is viable to envisage that Cyr61 expression can predict which of the ANN patients would benefit from the Tam adjuvant therapy. Therefore, we hypothesize that Cyr61 expression in breast tumor biopsies can predict the necessity of adjuvant therapy, the likelihood of relapse, and the response to antiestrogen therapy. This hypothesis will be tested in retrospective studies using archival breast cancer biopsies.				
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## INTRODUCTION

Breast cancer is a devastating disease that will affect an estimated one in eight women in an average lifespan. Of those women diagnosed with a primary breast cancer, up to 40% will develop metastatic disease. While we are yet unable to cure metastatic disease with current modalities, the antiestrogen Tamoxifen has been shown to produce long-term remissions in these patients. It is a well-tolerated medication, and may in fact have beneficial effects on bone density and reduction of cardiovascular risk factors. Unfortunately, all patients eventually fail tamoxifen therapy by developing resistance to the drug. The response rate to tamoxifen in the metastatic setting varies from 50-75% in estrogen receptor positive (ER+) tumors. Although the presence of ER is employed to predict the hormone dependency of a tumor, the relationship with response to endocrine therapy is not absolute (not all the ER+ tumors respond to endocrine therapy).

Significant levels of estrogen receptor (ER) have been detected in more than 60% of human breast cancers, but at best only two-thirds of these ER+ tumors respond to endocrine therapy. Why this should occur is still unclear. We have previously demonstrated that Cyr61, an angiogenic factor, is involved in breast cancer progression and the acquisition of antiestrogen resistance. More importantly, we showed that Cyr61 is expressed in 30% of breast carcinomas, all of which are estrogen receptor-negative (ER-). These experimental studies have demonstrated a relationship between expression of Cyr61, estrogen independence, and tamoxifen-resistance.

The line of reasoning of our proposal is based upon the function of Cyr61 in distinguishing between those tumors that will be sensitive to or resistant to Tamoxifen, and in distinguishing between those patients that will benefit from adjuvant therapy. There are several independent aspects to this reasoning: First, there is the evidence that Tamoxifen administration to ANN breast cancer patients induces a substantial regression of the tumor, as well as increasing disease-free survival. Second, invasive breast carcinomas often are estrogen-independent and antiestrogen resistant. Lastly, Cyr61 expression correlates with lack of estrogen receptor expression, hormone independence and antiestrogen resistance.

## **BODY:**

Cyr61 expression in breast biopsies associates clinically with HRG, ER and PgR and may predict response to anti-E2 and need of adjuvant therapy. Prediction of breast cancer recurrence can be assessed based on the size of the tumor, number of lymph nodes involved, ER and PgR status, and histological grade. It is known that adjuvant therapy improves long-term survival in breast cancer patients. The present therapeutic goal is to identify appropriate adjuvant regimens for different groups of patients, to reduce the toxicity and improve the efficacy of the drugs. Axillary node-negative (ANN) breast cancer patients have a good prognosis, however there is still an appreciable relapse rate. Adjuvant treatments will benefit some patients with ANN disease. However, large numbers of women (including those in whom no recurrence will occur) must be treated to benefit those destined to relapse. It is important to improve our predictions regarding which group of patients will benefit from these treatments.

A critical point of our proposal resides also in the function of Cyr61 in determining sensitivity vs. resistance of breast cancer to Tamoxifen (Tam). The administration of Tam to patients with ANN breast cancer induces a substantial regression of the tumor and an increase in disease-free survival. These data will clarify the variable responses of breast cancer patients to Tam and elucidate the role of Cyr61 in breast cancer. This information will be invaluable in understanding resistance/sensitivity to Tam, and might also allow us to use them as markers for a better prediction of therapeutic decisions. The data will be correlated with prognostic markers and clinical history. Specifically, we will assess whether expression of Cyr61 correlates with stage, grade, HRG, erbB-2 ER, and PgR status, disease-free survival, metastasis, relapse, and overall survival.

The original aims of the proposal were to examine expression of Cyr61 in histological subtypes of breast cancer tumors: To determine expression of Cyr61, we will use an anti-Cyr61 antibody (Ab) for immunohistochemistry (IHC). The detection of Cyr61 in breast cancer will provide a predictive/prognostic tool. This information could be of significance in intraductal, versus invasive, breast cancer and in lymph node-negative vs. positive patients. We will determine the significance of Cyr61 expression in two main groups of specimens:

- For understanding the prognostic value of Cyr61 expression in node-negative patients and to determine the role of Cyr61 in identifying those patients likely to recur—
- In defining the overall role of Cyr61 expression in invasive vs. intraductal tumors—  
These analyses will examine the joint variation of Cyr61 and HRG.

## **KEY RESEARCH**

### **1: Cyr61 (mRNA and protein) is overexpressed in HRG-positive and ER-negative breast cancer cells.**

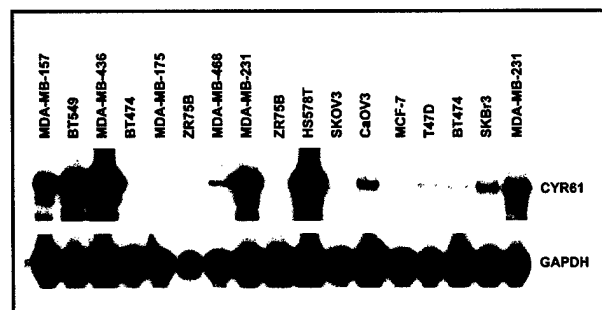
We first wanted to assess the expression of Cyr61 and the ability of an anti-Cyr61 antibody to stained paraffin embedded sections. This was accomplished using a number of breast cancer cells by RNase protection and Western blot analyses. Since the murine Cyr61 expression was regulated by serum, to ensure accurate measurements of Cyr61 expression, we cultured the cell lines under serum-depleted conditions, otherwise stated. Using RNA protection assays, the levels of Cyr61 were measured in RNA isolated from breast cancer cells. Since Cyr61 is a secreted protein, its expression was measured in conditioned media derived from breast cancer cells by Western blot analysis using an anti-Cyr61 polyclonal antibody (kindly provided by Dr. Lester Lau, at UIC). As can be seen in Figures 1A and 1B, and summarized on Table 1, Cyr61 is expressed in many breast cancer cells at the mRNA and protein levels.

Our studies demonstrate that Cyr61 overexpression correlates with HRG overexpression and with lack of ER expression, and correlates with the aggressive phenotype of the breast cancer cells, *i.e.*, their ability to invade, and to metastasize *in vitro* and *in vivo*. Of note, in serum containing media the T47D mRNA and the adjacent sample of BT474 mRNA, express higher levels of Cyr61 as compared with the same cell lines in the absence of serum (fourth lane from left). From this observation, it would appear that Cyr61 is not only regulated by serum (as shown in the mouse model) but it is possible that another component in the serum, besides E2, induces its regulation. Given the redundancy apparent for many cellular functions, some cells may not require Cyr61 to perform its functions, or may be selective in the Cyr61 functions they require. We have recently shown that Cyr61 is regulated by E2 in breast cancer cells.

## **REPORTABLE OUTCOMES:**

**Figure 1 A-B: Cyr61 is expressed in ER-negative and metastatic breast cancer cells.**

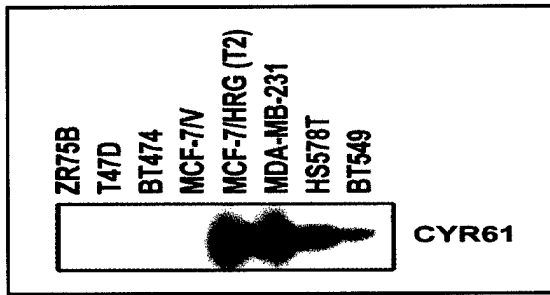
**A)**



Cells were cultured in IMEM containing 5% fetal bovine serum (FBS). Maintaining the cells in this manner does not pre select for populations later in the study. The medium was replaced by serum-free-medium (otherwise stated in the results) during a four-day period, to ensure complete depletion of serum for four days. Total RNA was isolated for analysis by

RNase protection assay. Protection assays were performed using 40  $\mu$ g of total RNA isolated from the different breast cancer cells. The loading was monitored by GAPDH as control.

B)



Cells were maintained as described in A. When cells reached about 80% confluence, conditioned medium (CM) was harvested and heparin chromatography was performed. Elutions were performed with increasing concentrations of NaCl and fractions were collected. Protein concentration of fractions was determined for all the fractions at high salt

elution (0.9M NaCl), and Western blot analysis was performed using an anti-Cyr61 antibody.

**Table 1: Cyr61 and HRG are co-expressed, and overexpression correlates with E2-independence, antiestrogen-resistance and aggressiveness.**

Cell line	Cyr61	HRG	ER	Vimentin	Invasive in vitro	Metastatic in vivo
MCF-7	-	-	++++	-	<sup>-1</sup>	-
T47D	-	-	++	-	<sup>-1</sup>	-
BT474	-	-	++	-	<sup>-1</sup>	-
MDA-MB-175	-	+/- <sup>3</sup>	+	-	<sup>-1</sup>	-
ZR75B	-	+/- <sup>3</sup>	+	-	<sup>-1</sup>	-
MDA-MB-468	+	-	-	-	<sup>+2</sup>	-
SKBR-3	+	-	-	-	<sup>+2</sup>	-
MDA-MB157	++	++	-	++	+	ND
MDA-MB-436	+++	+++	-	+++	+++	ND
BT-549	+++	+++	-	+++	+++	+
MDA-MB-231	++++	++++	-	+++	++++	+
HS578T	++++	++++	-	+++	++++	+

<sup>1</sup>Require E2 for invasion *in vitro* and growth *in vivo*, never metastasize *in vivo*. <sup>2</sup>Require ligand (EGF or HRG respectively) to invade, never metastasize *in vivo*. <sup>3</sup>E2 induces the expression of HRG. ND: Not determined

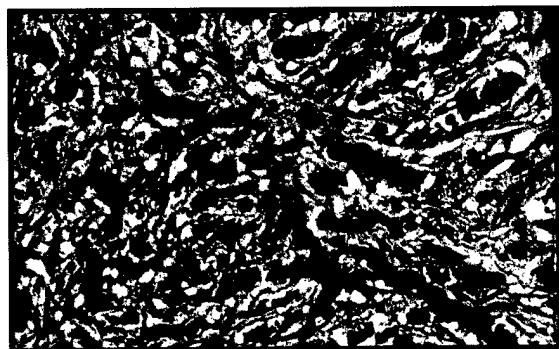
## **2. Cyr61 is expressed in 30% of breast cancer tumor biopsies:**

To determine whether the Cyr61 expression observed in our cellular models might have broader relevance, we investigated Cyr61 expression in human breast cancer biopsies. We considered these results a more logical next step than further cell line studies, such as transfection, since those studies are time-consuming and would be rendered largely irrelevant for breast cancer if we could not detect Cyr61 expression in the human disease. We initially examined 10 breast tumor biopsies by Western blot analysis, detecting strong immunoreactivity in 4 out of 10

samples (Figure 2B). Recently, we developed an immunohistochemical assay (IHC) for the detection of Cyr61 in paraffin-embedded sections. Our preliminary IHC analyses, performed on only a small number of cases (n=55), demonstrate that Cyr61 is expressed in 30% of invasive breast carcinoma, while it does not appear to be expressed in normal human breast tissue (Figure 2A).

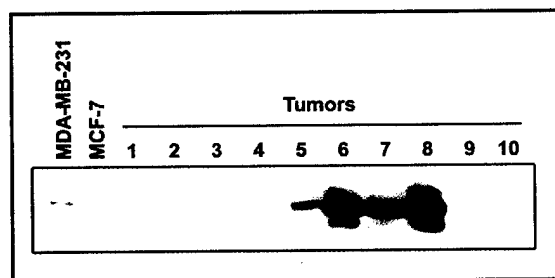
**Figure 2A-B: Cyr61 expression is detected in 30% of breast cancer biopsies.**

**A.**



Expression and localization of Cyr61 is detected, by IHC staining, in paraffin-embedded specimens, obtained from a breast carcinoma. The multistep detection system, Avidin-Biotin-Peroxidase method, was used. The tissue was deparaffined and rehydrated with xylene and alcohol. After a 30 min. treatment with blocking solution (goat serum 10% in PBS), an anti-Cyr61 antibody was applied for 1 hour (5 µg/ml).

**B.**



Tumors were lysed and equal amounts of protein were loaded on a 4-20% SDS-PAGE. Blots were visualized using an anti-Cyr61 antibody.

**3. Expression of Cyr61 in Invasive Breast Carcinomas:** In defining the overall role of Cyr61 expression in invasive vs. intraductal tumors. *These analyses will examine the joint variation of Cyr61 and HRG.* We have already stained one hundred samples for Cyr61 expression, and we will study in total more than 300 specimens. Correlations with known prognostic markers such as stage of progression, heregulin (HRG) and ER expression was also determined. We tested the expression of Cyr61 in invasive breast carcinomas and determined that it is expressed in 28% of breast carcinomas and it is co-expressed with HRG and inversely correlated with ER expression as shown in Table 2.



**Table 2: Cyr61 and HRG are co-expressed in invasive breast carcinomas (n100)**

	<b>Cyr61</b>	<b>HRG</b>	<b>ER</b>
<b>Cyr61</b>	<b>28%</b>	32%	3%
<b>HRG</b>	<b>32%</b>	30%	4%
<b>ER</b>	<b>3%</b>	4%	3%

**CONCLUSIONS:**

These data provide considerable evidence that Cyr61 may play an important role in breast cancer. However, the number of samples tested was too low to propose its predictive and/or prognostic relevance. No additional clinical information was available on these specimens; thus, additional studies are necessary to establish the magnitude and the impact of Cyr61 expression in breast cancer. Our previous data reveal that Cyr6 expression represents a critical factor in invasive breast carcinomas, and this is consistent with the idea that expression may be an important event in carcinogenesis. MCF-7 cells exhibit an early phenotype *in vivo* (noninvasive, non-metastatic, antiestrogen and hormone-responsive), because these cells are derived from a metastatic lesion, they express low to undetectable levels of Cyr61. The cell culture and the human biopsy data are consistent with Cyr61 playing an important role in HRG induction of breast cancer progression from an E2-dependent to an E2-independent and antiestrogen resistant phenotype. We have determined that Cyr61 expression correlates with HRG expression and inversely correlates with ER status. The expression of Cyr61 is extremely significant and will provide an additional tool into breast cancer diagnosis and prognosis.

The immunohistochemical study is exploratory, but will determine the likely incidence of detectable Cyr61 expression and the range of intensity. The ER/HRG study will determine whether large scale testing is warranted. Our approach should enable us clearly to establish the predictive/prognostic power of Cyr61 expression and any association among Cyr61, HRG, ER, and PgR expression in breast carcinomas.

**REFERENCES**

N/A

**APPENDICES:**

N/A